SHORT TERM RELEASE AND MECHANICAL STRENGTH OF FLUORESCEIN-IMPREGNATED CALCIUM SULFATE BEADS

UNDERGRADUATE HONORS THESIS

Presented in partial fulfillment of the requirements for a Bachelor of Science with Honors Research Distinction in Biomedical Engineering from The Ohio State University

By

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Abstract

This project is part of a larger project that involves juxtaposing antibiotic elution rates from two common bone cement beads, poly(methyl methacrylate) (PMMA) and calcium sulfate ($CaSO_4$). The beads are impregnated with the antibiotics vancomycin and tobramycin alone or in various combinations. In an attempt to preserve a primary joint or prevent a possible second infection after revision of a total joint arthroplasty, PMMA and $CaSO_4$ beads are commonly infused with antibiotics because of their ability to elute locally high concentrations of them. By investigating this juxtaposition, it can be determined which cements' antibiotic elution rate may be optimal on a patient-to-patient basis in treating a simulated bacterial infection *in vitro*.

In previous studies, antibiotic-infused beads were placed onto an agar plate inoculated with bacteria in order to determine zone of inhibition (ZOI) of the lawn caused by the antibiotic elution. However, this does not necessarily correlate with medical practice as beads are utilized once a biofilm infection has already been established. Thus, our proposed experimental method simulates a clinical application of these beads being placed where biofilms may already be present. Lawns of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were established onto separate plates for 24 hours, and then the beads are introduced to determine their respective zones of killing (ZOK). Bioluminescent strains were used in order to track shutting down metabolic

activity and killing of the lawn biofilms in real time. PMMA's antibiotic elution rate and spread of ZOK were compared to the $CaSO_4$ beads.

In this project, we aligned the ZOK assays with the diffusion of antibiotics by using fluorescein-infused PMMA and $CaSO_4$ beads to analyze its diffusion characteristics through an agar plate. Fluorescein, commonly utilized as a fluorescent tracer, is more economical and much easier to track with a gel documentation system than tobramycin and vancomycin. Because this therapeutic method is specific to the patient, the surgeon may impregnate the beads with different amounts of antibiotic. Often times, surgeons will use concentrations outside of the range of manufacturer suggestions, which varies the mechanical strength of the beads. Because any cement additive will influence microstructure, and subsequently mechanical strength and release kinetics, the weights and first cracking strengths of the beads were correlated to varying concentrations of fluorescein impregnation. We determined that with high concentrations of fluorescein, although the beads locally elute at a higher rate, their mechanical efficacy is seriously hindered.

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I would like to heavily thank Dr. Stoodley for his willingness to give me the opportunity work in his group without any prior research experience. Learning the research process, getting up to speed on the projects in his lab, and formulating my own experiment for an undergraduate honors thesis all in one year was tough. Dr. Stoodley helped me heavily through the process, and went above and beyond for me in every facet to assure it was smooth. I am extremely grateful for his time and effort, and I hope to continue a professional relationship with him in my career. He has great passion for his work and I hope other undergraduates have the luxury of working in his lab group in the future.

I would like to thank Dr. Granger for allowing me to shadow one of his total knee explant surgeries and for introducing me to Dr. Stoodley.

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Poster Presentations

Knecht, C., Diamond, S., **Farrar, N.**, Peters, C., Swearingen, M., Cooper, J.J., Aiken, S.S., Granger, J., Howlin, R.P., Stoodley, P. *Antibiotic release from absorbable calcium*

sulfate beads for the prevention and treatment of biofilm formation in orthopaedic prosthetic infections. EUROBIOFILMS 2015 4th European Congress on Biofilms, ESCMID Study Group for Biofilms. Brno, Czech Republic. June 23-26 2015.

Farrar, N., Knecht, C., Diamond, S., Peters, C., Swearingen, M., Granger, J., Howlin, R.P., Nocera, T., Stoodley, P. *Short Term Release from Calcium Sulfate and Polymethyl Methacrylate Beads*. Poster presented at 2015 National Conference on Undergraduate Research, Eastern Washington State University, Spokane, WA. April 16-18 2015.

Farrar, N., Knecht, C., Diamond, S., Peters, C., Swearingen, M., Granger, J., Howlin, R.P., Nocera, T., Stoodley, P. *Short Term Release from Calcium Sulfate and Polymethyl Methacrylate Beads*. Poster presented at 2015 Denman Undergraduate Research Forum, The Ohio State University, Columbus, OH. March 25 2015.

Farrar, N., Knecht, C., Diamond, S., Peters, C., Swearingen, M., Granger, J., Howlin, R.P., Nocera, T., Stoodley, P. *Short Term Release from Calcium Sulfate and Polymethyl Methacrylate Beads*. Poster presented at 2014 Sigma Xi International Research Conference, Glendale, AZ. November 7-8 2014.

Fields of Study

Major Field: Biomedical Engineering, Pre-medicine

Minor Field: Entrepreneurship

Table of Contents

List of Figures

List of Tables

1. Introduction

Infection is a major complication associated with total joint arthroplasty and other orthopedic implants. The formation of bacterial biofilm is of particular concern as it is difficult to diagnose and can lead to chronic infections. Transitioning from planktonic organisms and adhering to implants, bacteria grow and divide into communities.[i] This becomes a bacterial biofilm when these sessile communities of cells secrete an extracellular polymeric slime matrix that is resistant to standard antibiotic therapy.[ii] As a result of biofilms and their ability to spread, millions of people each year experience chronic infections (Figure 1).[iii,iv]

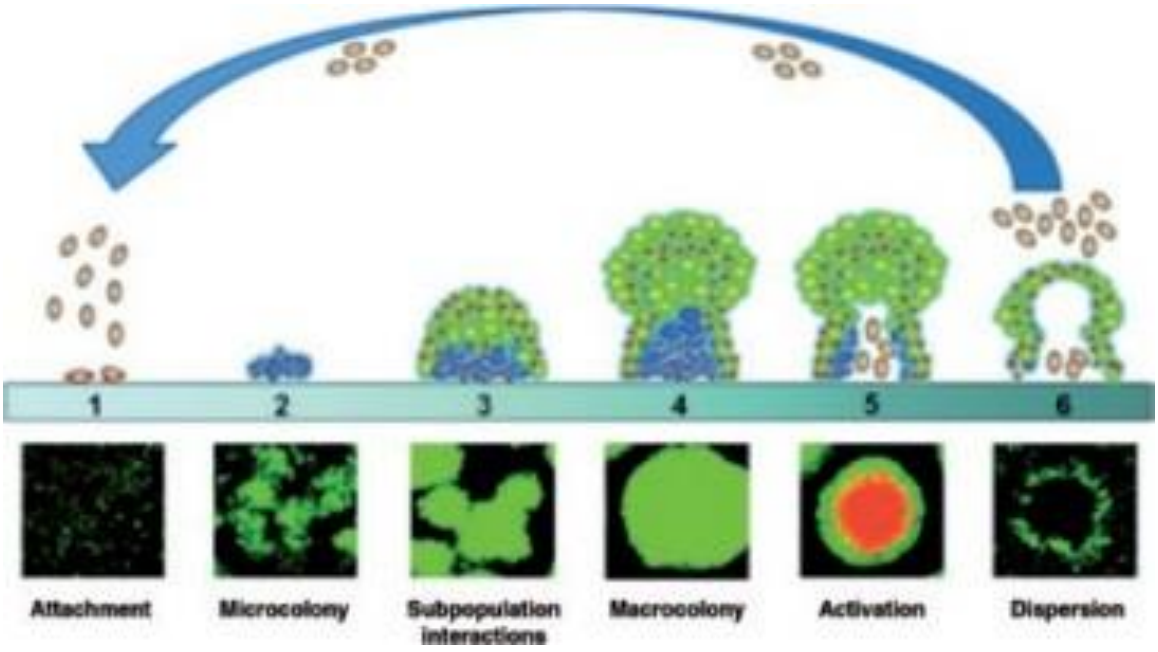


Figure 1: Figure of the attachment, colonization, and activation associated with biofilm development and dispersion. Biofilm can adhere to biological and non-biological surfaces, and disperse throughout the body creating a chronic infection.

The effectiveness of conventional antibiotics is restrained due to resistance and the difficulty of culturing the bacteria to determine the species causing the infection.[v] Bacterial infections are reported in up to 24% of total joint replacement surgery, making them a significant problem.[vi] If the infection becomes chronic, removal and replacement of the joint is required, resulting in a total knee explant surgery.[vii]

In an attempt to preserve a primary joint or prevent a possible second infection after revision, nonabsorbable polymethyl methacrylate (PMMA) cement and absorbable cements such as calcium sulfate beads can be antibiotic-loaded to fight bacterial infections, and placed into the site of suspected infection as vessels. PMMA and $CaSO_4$ beads are commonly infused with antibiotics such as tobramycin and vancomycin because of their ability to elute locally high concentrations of antibiotics in an attempt to clear an infection and inhibit biofilm formation following joint replacement surgery. Common bacterial infections in total joint arthroplasty surgeries include *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*, which are respectively susceptible to tobramycin and vancomycin.[viii] In cases where the surgeon is unable to identify the type of bacteria causing the infection, they will impregnate the beads with both antibiotics. PMMA is used in a structural role to stabilize the implant in bone tissue or provide an articulating spacer. $CaSO_4$ assists in the regeneration of bone, but is not used for structural integrity due to its weaker strength. Stimulan, a high purity synthetically derived $CaSO_4$ has recently been used as a drug delivery vehicle in orthopedics. Stimulan has the advantage that there are less adverse reactions in patients than found with cements purified from mined mineral.

Surgeons understand bone cements elute antibiotics that can inhibit and destroy biofilm formation; however, the elution kinetics and microstructure variance as a result of antibiotic-impregnation has not been fully investigated. Because antibiotic-loaded cement methods are specific to the patient, the surgeon may impregnate the beads with a concentration of antibiotic of their choosing. This variation inevitably alters the microstructure of the beads, and subsequently may hinder their mechanical strength and release kinetics. Furthermore, with high concentrations of antibiotics, cytotoxicity may become an issue. Thus, the main goals of this investigation are to determine what effects varying the concentration of antibiotic in the PMMA and Stimulan beads and the media in which the antibiotic is diffusing through has on the efficacy of their respective structural and regenerative purposes, elution kinetics, and mechanical strength.

The hypothesis is that antibiotic-impregnation, especially at high concentrations, alters and weakens the microstructure of the beads, decreasing their efficacy**.** If the antibiotics impregnated in the beads can alter their mechanical strengths, potentially due to an increase in porosity of the molecular structure, their elution rate and their mechanical and regenerative roles, respectively, may be hampered. Furthermore, although the diffusion coefficients of molecules in biofilm have been found to be half of that in standard agars, the release rates from a cement bead has not been investigated, and are hypothesized to decrease. [ix,x]

Fick's second law predicts the rate of change of concentration within a media as a function of time. It simulates unsteady-state molecular diffusion through a media. From this law, the Einstein-Smoluchowski proportionality relationship can be derived from,

$$d = \sqrt{2Dt} \quad \text{(Eq. 1)}$$

$$d \propto \sqrt{t} \quad \text{(Eq. 2)}$$

where d, D, and t represent distance, the diffusion coefficient, and time, respectively.[xi] Because antibiotic elution from an impregnated bead through a media is an example of unsteady-state molecule diffusion, Fick's second law can be administered to model elution distance as a function of time. In this study, power law relationships were utilized to analyze the data because of this proportionality relationship.

In quantifying the alteration of the microstructure of Stimulan and PMMA beads upon mixing with an additive, the compression-induced failures of various combinations of antibiotic-impregnation were investigated. Fluorescein, a fluorescent tracer utilized to tag antibiotics, is more economical and easier to track than antibiotics because it emits fluorescence under ultraviolet rays; thus, it was utilized to model the elution profiles from Stimulan beads in this study.[xii] Moreover, Stimulan beads were impregnated with increasing concentrations of fluorescein to determine whether varying the alteration of the microstructure would hinder their intrinsic elution ability and structural integrity. By determining the alteration of elution distance as a function of structural integrity, a correlation between release rates and mechanical deficiency was created. Finally, to determine whether biofilm inhibits elution rate, fluorescein elution from Stimulan was measured in media with a pre-grown lawn. This understanding will assist surgeons in determining if their current surgical practices are efficacious on a patient-to-patient basis, and researchers in choosing which media is the most physiologically relevant for the elution of impregnated bone cement.

2. Materials and Methods

2.1 Fabrication of Stimulan and PMMA beads

2.1.1 Antibiotic Impregnation

All of the experiments conducted required the fabrication of Stimulan (Biocomposites, Ltd.) and PMMA (Palacos® R, Zimmer Holdings) beads with or without impregnation. In Biocomposites, Ltd. mixing guide for Stimulan, it is recommended to infuse 1000 mg of powder antibiotic, such as Vancomycin (Sigma-Aldrich) and Tobramycin (Sigma-Aldrich), to ensure the most efficient set time.[xiii] Because the first experiment was conducted to simply quantify the effects of a microstructure alteration of the beads separately, the weight of antibiotic infused into PMMA was normalized to Stimulan on a wt.% per bead basis. Infusing 1 g of antibiotic into one 10 cc pack of Stimulan resulted in a 4.2 wt.% of antibiotic, which when normalized to PMMA, resulted in a need of 2.23 g of antibiotic per pack (Figure A1). The Stimulan and PMMA beads were fabricated with three unique antibiotic combinations—tobramycin, vancomycin, and tobramycin and vancomycin. When both antibiotics were impregnated, there was 4.46 g of total antibiotic added, and impregnated beads were compared to non-impregnated beads. The mixing instructions in Figure 2 were used for the antibiotic impregnation.[xiv]

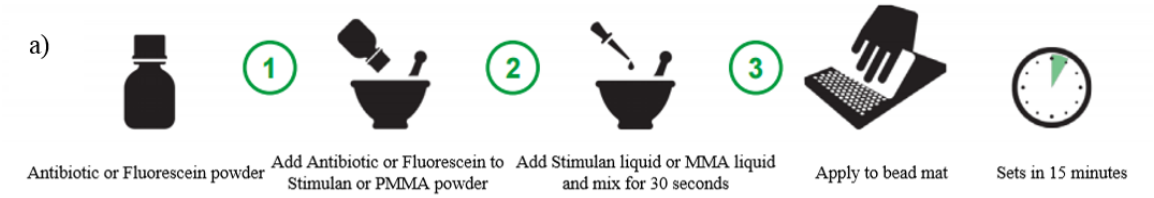
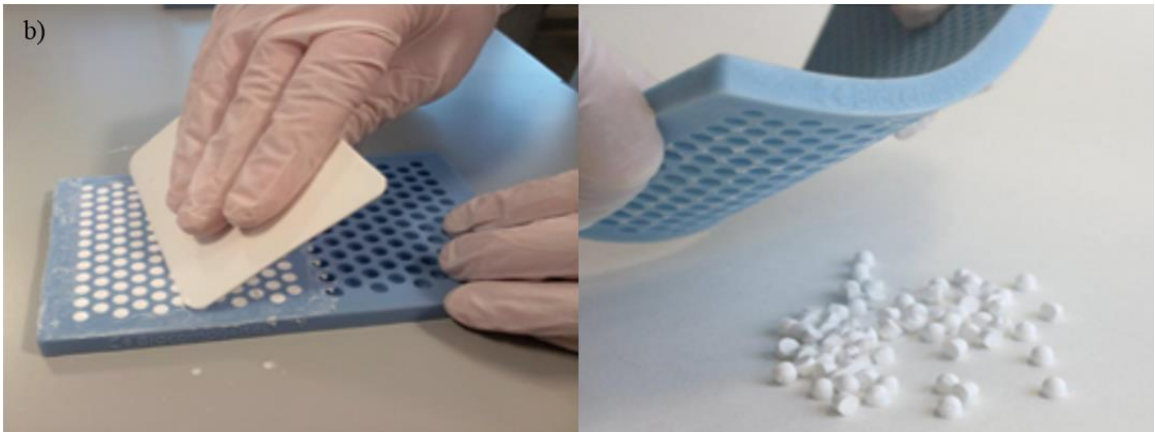


Figure 2: (a) Step-by-step instructions for the mixing of Stimulan and PMMA beads with and without antibiotics. In order to formulate the control beads, step 1 was omitted. A Stimulan pack contains 20 g of powder and 6 mL of liquid. A Palacos® R pack contains 40 g of PMMA powder and 20 mL of MMA liquid.



(b) Image of spreading of the Stimulan or PMMA beads onto the bead mat. This highlights step 3 from Figure 2a. After setting for 15 minutes, the beads are extruded.

2.1.2 Fluorescein-Impregnation of Stimulan Beads

In order to investigate the effect of varying the degree of microstructure alteration in Stimulan beads, they were impregnated with 7 different concentrations of fluorescein, 0 g, 0.125g, 0.25 g, 0.5 g, 1 g, 2 g, and 4 g, with 0 g acting as the control. In order to not waste supplies, approximately only 10% of a pack was formulated for each concentration. The weight of fluorescein to be impregnated was added to the set weight of one pack of Stimulan materials, 23.76 g. From Table 1A, the amount of $CaSO_4$ powder, Stimulan liquid, and fluorescein necessary to achieve each of the desired concentrations was obtained. The powder was weighed in a weighing dish, and the corresponding liquid was

pipetted into the mixing dish. The weight of fluorescein on a per bead basis is contained in Table 2A. The beads were formulated by implementing the method in Figure 2.

2.2 Mechanical Testing

In measuring the structural integrity of the bone cements beads before and after impregnation, a compression test was administered. The bead under investigation was placed between two parallel plates and crushed with a constant strain rate (Figure 3). The

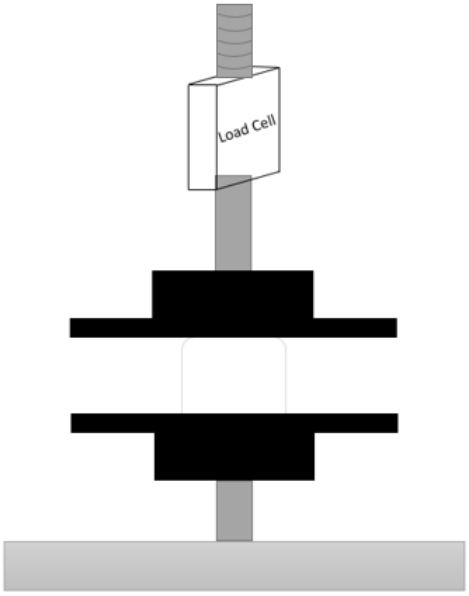


Figure 3: PowerPoint composed assembly of compressive test profile frame. The load cell measures the frame's current applied force from the shaft above it. The compression plates crush the bead with a constant positional change (1 mm/min) until the bead exhibits a crack. A Stimulan bead is 4.8 mm in height, thus the strain rate the bead experienced is 0.208/min.

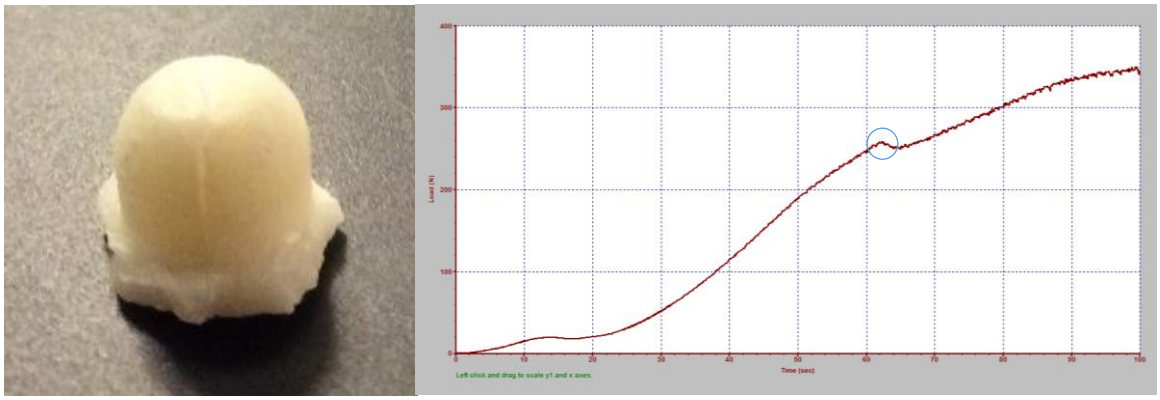
test frame (100Q Series Mechanical Test Frame, Test Resources) was activated by turning on the device and running a compressive test profile with a constant positional change in the parallel plates. In optimizing the strain rate, 1 mm/min was selected due to the uniformity and consistency of the load (Newtons) as a function of time (seconds) graphs produced from the pre-trials. The top compression plate was manually jogged

until it was flush with the top point of the spherical section of the bead, and this position was set to 0. Upon initiation of the constant strain the threaded top shaft was forced into the apex of the bead, and the force was measured in real-time by the load cell. The plots of load as a function time were transmitted from the test frame to a laptop using WinCom™.

The graph of load as a function of time for a Stimulan bead, exhibited a clear first crack point, which was termed its ultimate compressive strength (Figure 4a).

Figure 4: (a) Image of crack propagation down the center of a Stimulan bead (left). The ultimate compressive strength is clearly indicated by a severe drop of the compressive applied force in the plot of load as a function of time (right).

Contrastingly, in the preparation of PMMA, the MMA liquid initiates a polymerization reaction with the PMMA powder when added, creating a polymer chain that has the ability to plastically deform. Thus, the applied force recorded was when the microstructure of the bead began permanent deformation—termed the first crack strength (Figure 4b). Maximum load values of the impregnated beads were exported to excel and compared to their respective control beads.



(b) Image of crack propagation down the center of a PMMA bead (left). The ultimate compressive strength is clearly indicated by a severe drop of the compressive applied force in the plot of load as a function of time (right).

2.3 Media and Lawn Preparation

2.3.1 1% Agar Preparation

1% Agar growth media was used to as the diffusion substrate for fluorescein-impregnated Stimulan beads with increasing concentrations. To produce 1% Agar, 100 mL of stock 10x phosphate buffered saline (PBS) solution was diluted to 1000 mL of 1x with dH_2O in a flask. 10 g of Agar was weighed and stirred into the flask with a magnetic stir rod on a hot plate stirrer. When the solution began to boil, it was removed and poured into 40 plates with an outer, lower cap diameter of 87.5 mm. The plates were cooled at 4°C for 12 hours.

2.3.2 LB Agar Growth Media Preparation and Lawn Growth

To determine whether biofilm inhibits the elution of fluorescein from a Stimulan bead, the elution distance of fluorescein from a Stimulan bead through a lawn of PA01 Xen41 *Pseudomonas aeruginosa* in LB Agar growth media was compared to that of LB Agar growth media with no pre-grown lawn. In producing LB Agar growth media, 1000 mL of dH_2O and 37 g LB Agar were mixed. The solution was then autoclave-sterilized

before growing a lawn on it, and poured into 40 plates. Lawn spreads were created by pipetting 5 mL of stock LB Broth into a tube and mixing it with one dab of thawed Xen 41-strain *P. aeruginosa* (-80°C). The culture was placed in a rolling incubator (37°C) for 24 hours to grow. After the culture was removed, it was diluted to 1% with LB broth, per the laboratory's protocol, and spread across the LB Agar growth media. For the control trials, pure LB broth was spread across the plates. All plates were placed in an incubator for 24 hours. Lawns across the plates were formed (Figure 5a), and their activity was confirmed with the photon counter setting on an IVIS imaging system (Figure 5b).

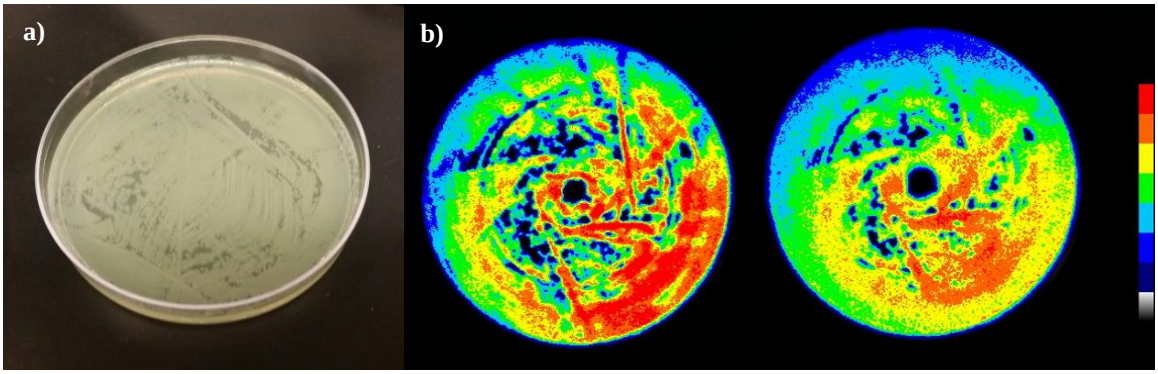


Figure 5: (a) Bioluminescent mage of lawn of *P. aeruginosa* on LB Agar growth media after 24 hours of incubation. (b) Images of bacterial photon emission representing the colony's activity. The IVIS imaging system counted the photons density across the lawn spread and displayed an image of the scaled bacterial activity—red indicating the most active regions. The left and right images represented the lawn after 24 and 32 hours, respectively. The black region in the middle of each plate corresponded to the fluorescein-impregnated Stimulan bead.

2.4 Gel Documentation and Incubation

Fluorescein-impregnated Stimulan beads were placed on their respective plates, and a gel documentation system (Gel Doc™ XR+ System, Bio-Rad) was utilized to capture a time lapse of elution from the beads. Nine fluorescein-impregnated Stimulan beads—seven different concentrations and two different media—were analyzed in determining the effect of infusion concentration on elution and of biofilm on elution,

respectively. All plates were manually exposed to a constant intensity of ultraviolet radiation (UV). Samples were incubated between the eight 1 hour intervals imaged.

Images were saved as JPEG files, and Fiji Imaging Processor (Fiji Is Just ImageJ, NIH) was utilized to calculate elution distances. Plots were created in excel to compare changes in elution over time when the fluorescein concentrations were fluctuated and when the media was varied, as well as in determining a correlation of fluorescein elution distance as a function of mechanical integrity. MATLAB (R 2013b, The Mathworks, Inc.) code was written to produce a 3D mesh of fluorescein elution as a function of time and concentration (Figure A2).

2.5 Image and Statistical Analysis

Elution experiments were done is triplicate, while six trials were used for the mechanical properties tests. For each trial in Fiji, nine time points—between 0 and 8 hours from bead placement—were aligned by plate and bead to create a set of images that was normalized in pixel size. The plate diameter measured 87.5 mm, and was used for the scale to pixel ratio setting. The 2D images were stacked on top of each other (Figure 6a) to create a 3D data set. A slice cut was administered to the stack (Figure 6b), and an automatic intensity threshold was applied to determine elution distance with consistent fluorescence (Figure 7c). The elution distance was determined by measuring the respective layers.

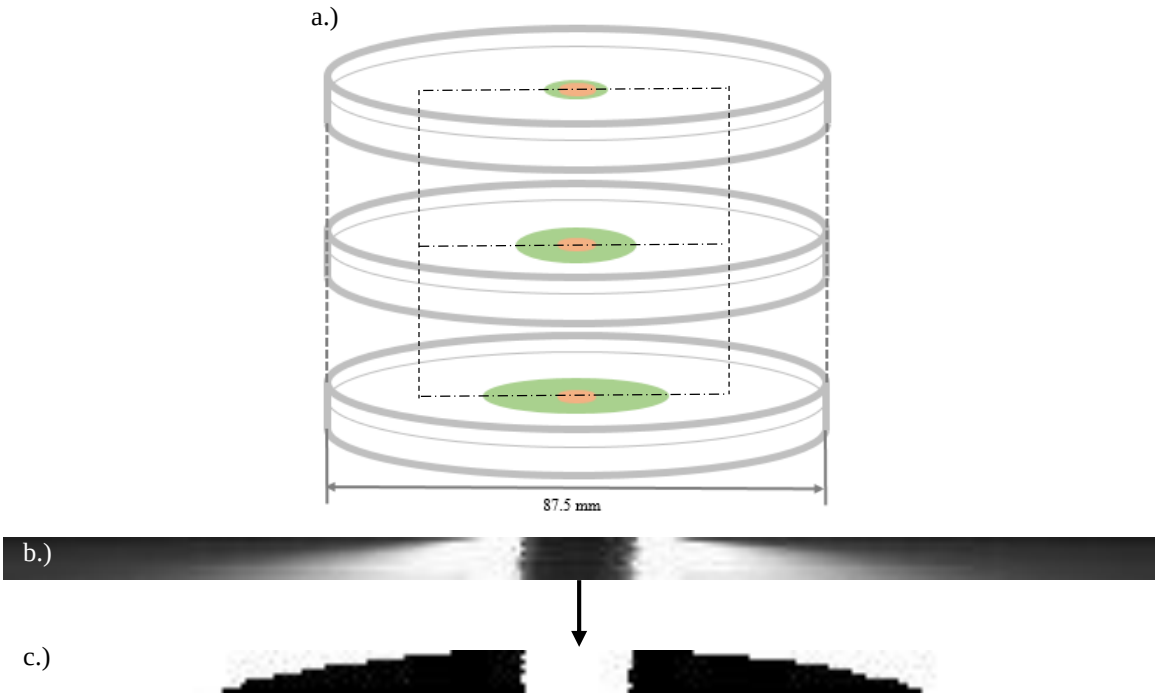


Figure 6: (a) Stack of plates in Fiji over an increasing length of time. The orange region represents the fluorescein bead and the green region represents its elution area. The slice cut went through the center of the bead and is represented by the black lines. (b) and (c) Slice cuts of a time lapse stack. From (b) to (c) a fluorescence intensity was selected that was consistent for all trials. In (c) there were 9 layers that were measured, one for each time point. The blank area in the center was the location of the bead. The image displays an inverted contrast to that of (b).

2.5.1 Statistical Analysis

Scatterplots, R^2 values, and trend lines were generated in excel. In all experiments, standard deviation were calculated and represented by error bars. Finally, one-tailed student t-tests were administered for unpaired data sets with equal variance, and 95% confidence intervals were computed.

3. Results

3.1 Additive-Induced Microstructure Alteration of Bone Cement Beads

Stimulan and PMMA beads were compressed until they exhibited their first crack on the plots of applied load as a function of time. The beads with impregnated antibiotics were plotted in bar chart and tested for a significant difference in structural integrity compared to their respective control beads.

Ultimate compressive strengths for Stimulan impregnated with tobramycin and vancomycin, tobramycin, and vancomycin, were juxtaposed to non-impregnated Stimulan in Figure 7. Upon impregnation, only vancomycin addition measured a

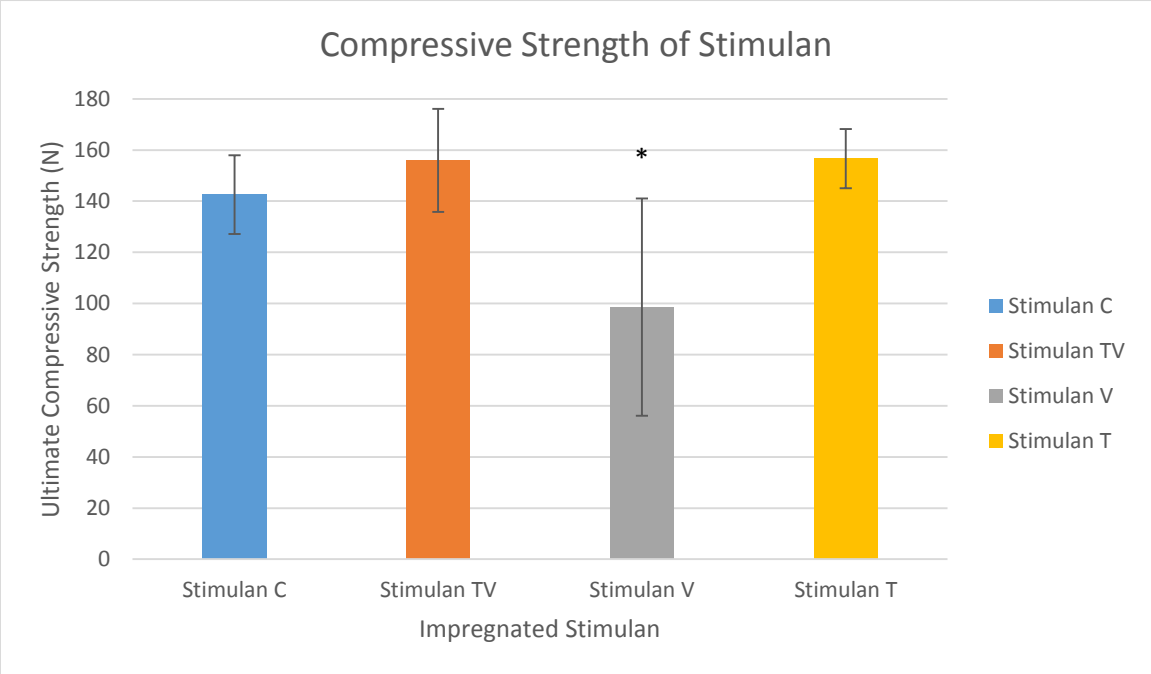


Figure 7: Ultimate compressive strengths data of Stimulan plotted with different antibiotic combinations to determine microstructure alteration. Ultimate compressive strengths were measured in Newtons. A

significant reduction in the strength of vancomycin-impregnated Stimulan can be seen (p = 0.049), indicated by "*".

significantly weaker microstructure (p = 0.049) than the control Stimulan bead. Microstructure weakening upon addition of tobramycin (p = 0.165) and tobramycin and vancomycin (p = 0.0963) was not statistically significant, and actually increased the bead's ultimate compressive strength. Although their standard deviations were much higher, when combining the data from the three different antibiotics to determine the overall effect of an additive on the strength of Stimulan, the alteration was determined to be not significant (p = 0.394).

First crack strengths for PMMA impregnated with tobramycin and vancomycin, tobramycin, and vancomycin, were juxtaposed to non-impregnated PMMA in Figure 8.

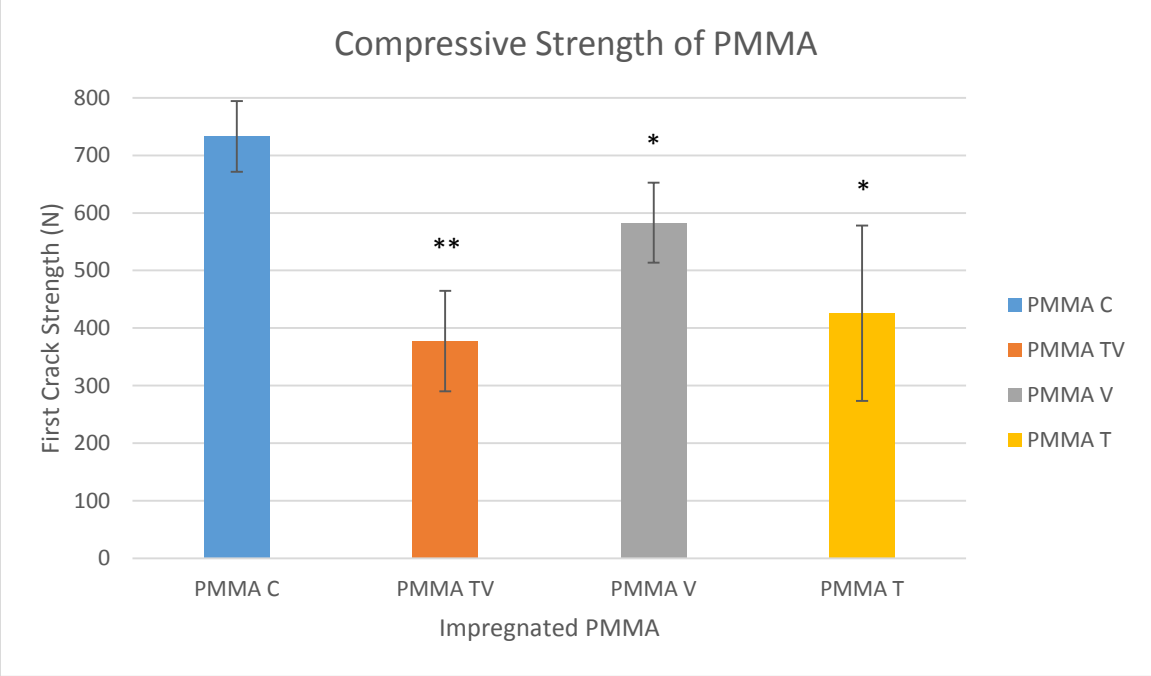


Figure 8: First crack strengths data of PMMA plotted with different antibiotic combinations to determine microstructure alteration. First crack strengths were measured in Newtons. A significant difference in the strength of the addition of one antibiotic in PMMA was observed (p = 0.000898). Furthermore, a significant difference in the strength of the addition of two antibiotics in PMMA was observed also (p =

0.00276). “*” represent one set of statistically similar results, and “**” represent another, weaker, set. Both sets are statistically different than the control.

Upon impregnation, all antibiotic additions measured a significantly weaker microstructure than the control PMMA bead ($p = 0.00276$; 0.00886; 0.00478, respectively). Beads with one added antibiotic were determined to be significantly weaker than control beads ($p = 0.000898$). Differences in first cracking strengths of the addition of two antibiotics and one antibiotic also resulted in statistically weaker beads ($p = 0.0419$). Not only were standard deviations of impregnated-PMMA beads higher than non-impregnated, they were overall much weaker ($p = 0.000232$).

3.2 Stimulan Structural Integrity

Fluorescein-impregnated Stimulan beads were compressed until their ultimate compressive strengths were observed on the plots of applied load as a function of time. Because the concentration of fluorescein was increased, a scatterplot of ultimate compressive strength as a function of concentration was able to be created. Student t-tests were performed on each of the concentrations of fluorescein, and compared to the non-impregnated Stimulan bead.

In figure 9, the ultimate compressive strengths of beads with 0, 0.125, 0.25, 0.5, 1, 2, and 4 g of fluorescein per 10cc pack of Stimulan were plotted. All concentrations of fluorescein were determined to have significantly different ultimate compressive strengths when compared to the control bead (Table 1), with the exception of 1 g of fluorescein per 10cc pack of Stimulan ($p = 0.088$). Initially, the Stimulan beads increased in strength up to an additive concentration of 0.5 g per 10 cc pack of Stimulan.

Concentrations of 2 g and above, resulted in a rapid weakening of the microstructure, and were difficult to mix.

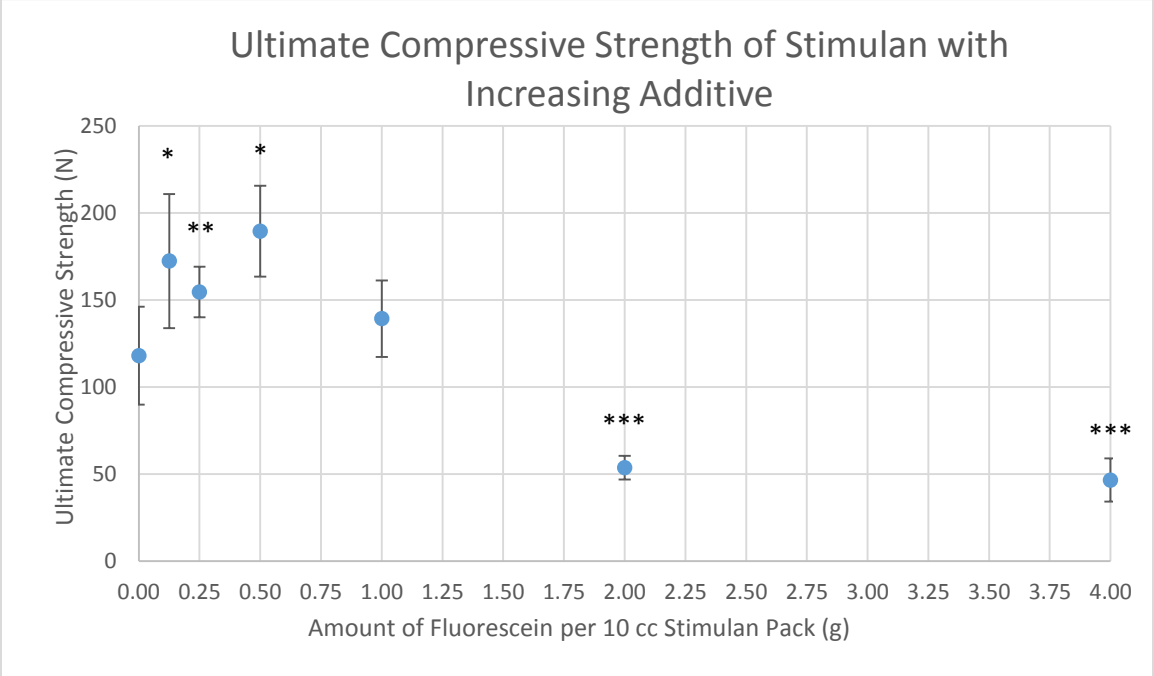


Figure 9: Ultimate compressive strength of fluorescein-impregnated Stimulan beads as a function of concentration. 1 g of fluorescein per pack of 10cc Stimulan showed a statistically similar Ultimate compressive strength to that of the control.

FLUORESCEIN-IMPREGNATED STIMULAN STATISTICS

Weight (g)	P
0.125	0.00959
0.25	0.00905
0.5	0.000523
1	0.0880
2	0.00114
4	0.000101

Table 1: P-values calculated by comparing different concentrations of fluorescein-impregnation of Stimulan beads and the non-impregnated fluorescein beads. All concentrations showed significant differences from the control in structural integrity except 1 g of Fluorescein per 10cc pack of Stimulan.

3.3 Stimulan Release Kinetics

After fluorescein-impregnated Stimulan beads were placed on 1% Agar plates, their elution distances were imaged by a gel documentation UV transilluminator camera. 15-30 minutes passed before all beads were placed on their respective plates and imaged. Hourly time points of the elution of a Stimulan bead impregnated with 4 g of fluorescein per 10 cc pack were comprised into Figure 10. It was clear that with an increase in time,

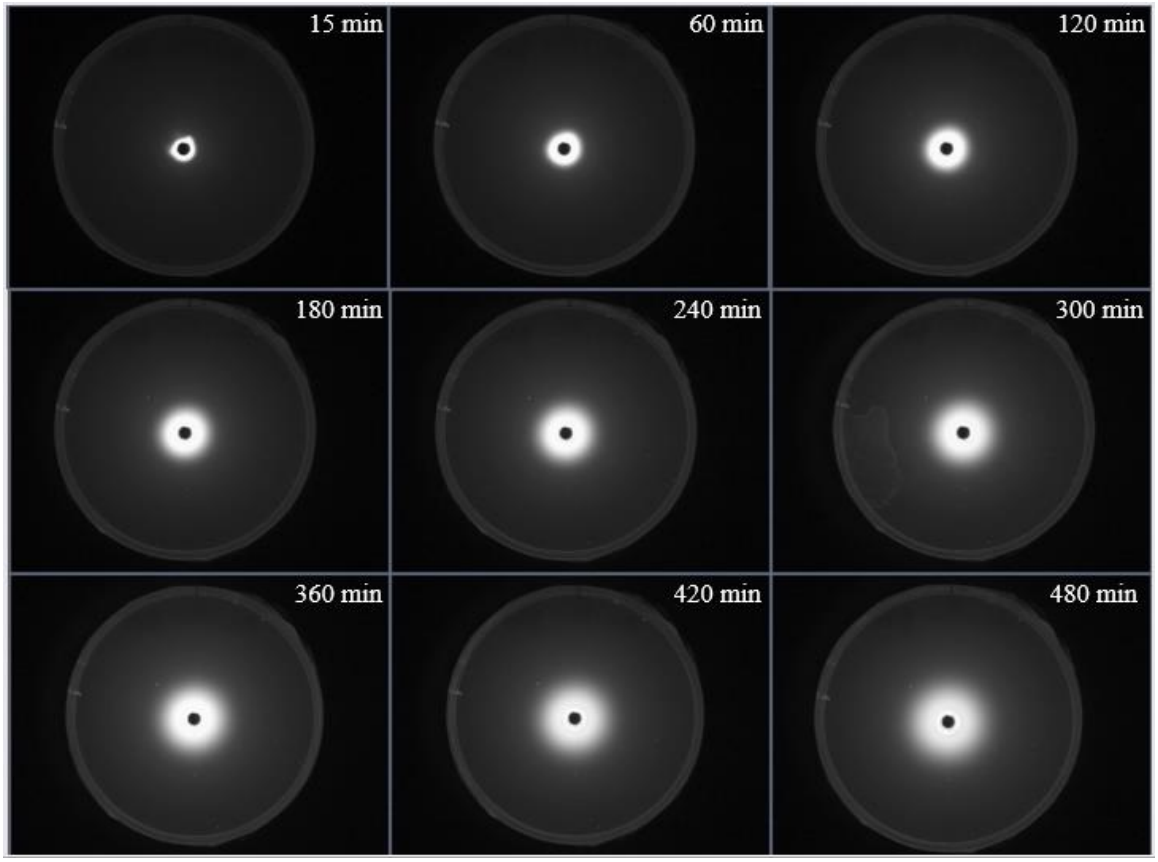


Figure 10: Images taken from the Gel Documentation system of the elution of fluorescein from a Stimulan bead. The images depict the time lapse of a Stimulan bead with 4 g of fluorescein per 10cc pack. The UV camera was capturing the elution area from the reflection of UV rays off of the fluorescein.

the elution distance of the bead across the media increased.

The elution distances were quantified in ImageJ, and plotted as a function of time for each concentration (Figure 11). Power law trend lines were implemented in representing the data because the elution profiles were compared to the half order release rate kinetics in Eq. 2. With every increase in additive concentration, and increase in elution distance was observed with respect to a change in time.

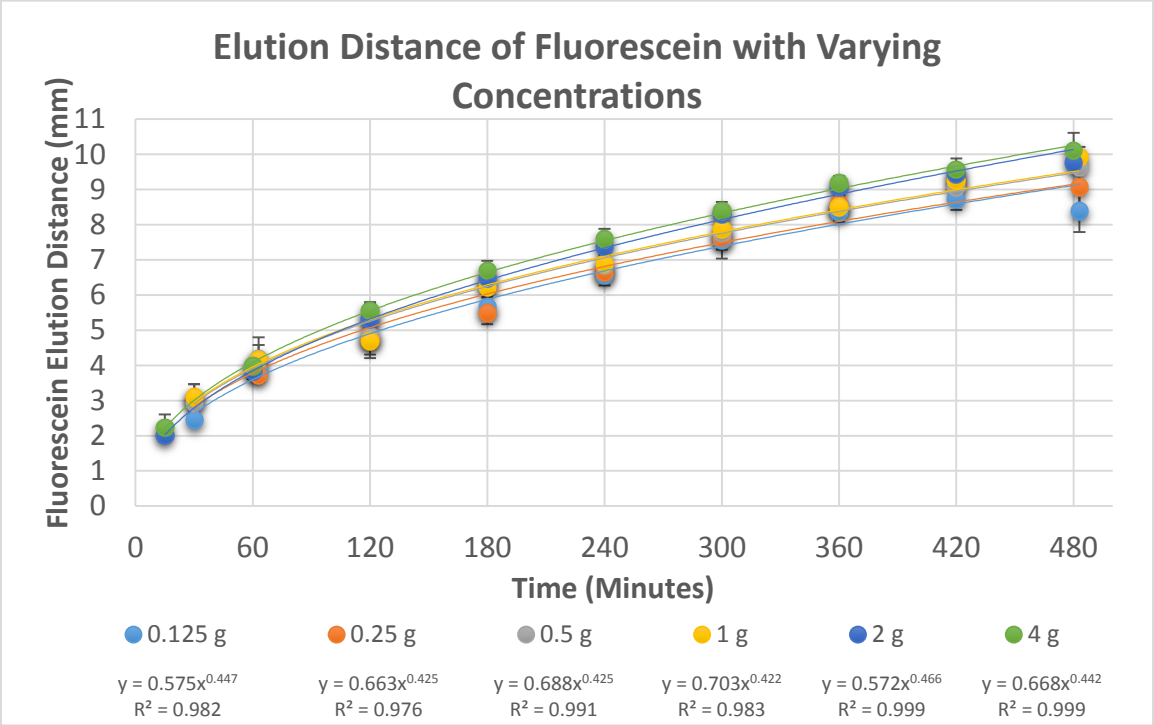


Figure 11: Plot of fluorescein elution distance as a function of time through 1% Agar. The elution distance increases with an increase in concentration of the bead. Each data point represents the mean of three trials. Power law trend lines were used represent the data because it should adhere to Fick's second law of half order kinetics.

The data from figure 11 was exported to MATLAB, and a mesh of the elution distance of fluorescein as a function of time and concentration was plotted (Figure 12).

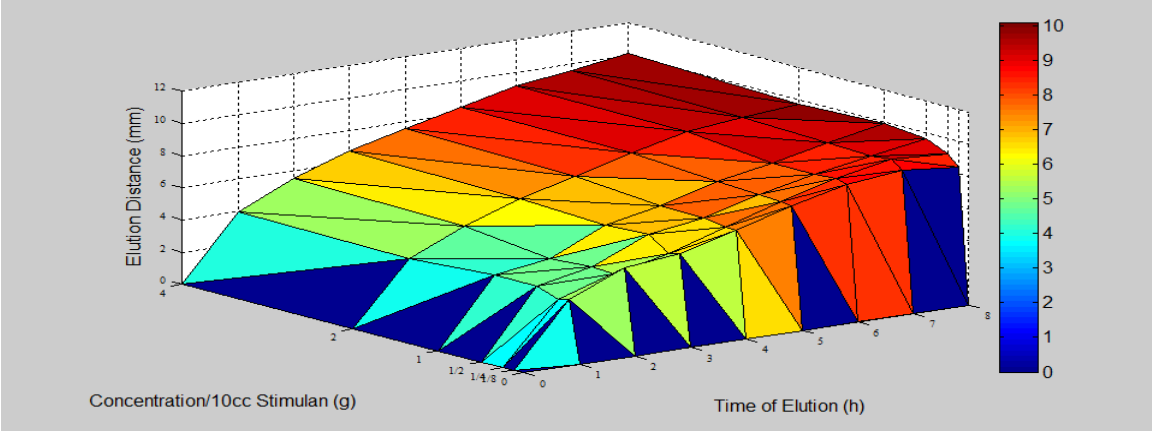


Figure 12: Plot of elution distance of fluorescein-impregnated Stimulan beads as a function of time and concentration produced by MATLAB. The colorbar on the right side represents the elution distance on the planes of the mesh. Each data point was plotted with an x-,y-, and z-coordinate.

From the plot, it was determined that by increasing the concentration of additive in a Stimulan bead, or the time in which a Stimulan bead was allowed to elute, the distance of elution increased with an initial burst, which slowed with higher concentrations and longer elution periods.

In determining if adding high concentrations of additive to Stimulan beads would significantly increase elution distance at respective time points, Figure 13 was produced. 4 hours and 8 hours were shown as examples because they were the half way and final points in the time lapse, respectively. Every increase in concentration resulted in a statistically significant increases in elution distance of fluorescein; thus, the elution ability of the beads was maintained with high additive concentrations. However, as is seen with the exponents of the power laws in Figure 13, the increases were miniscule.

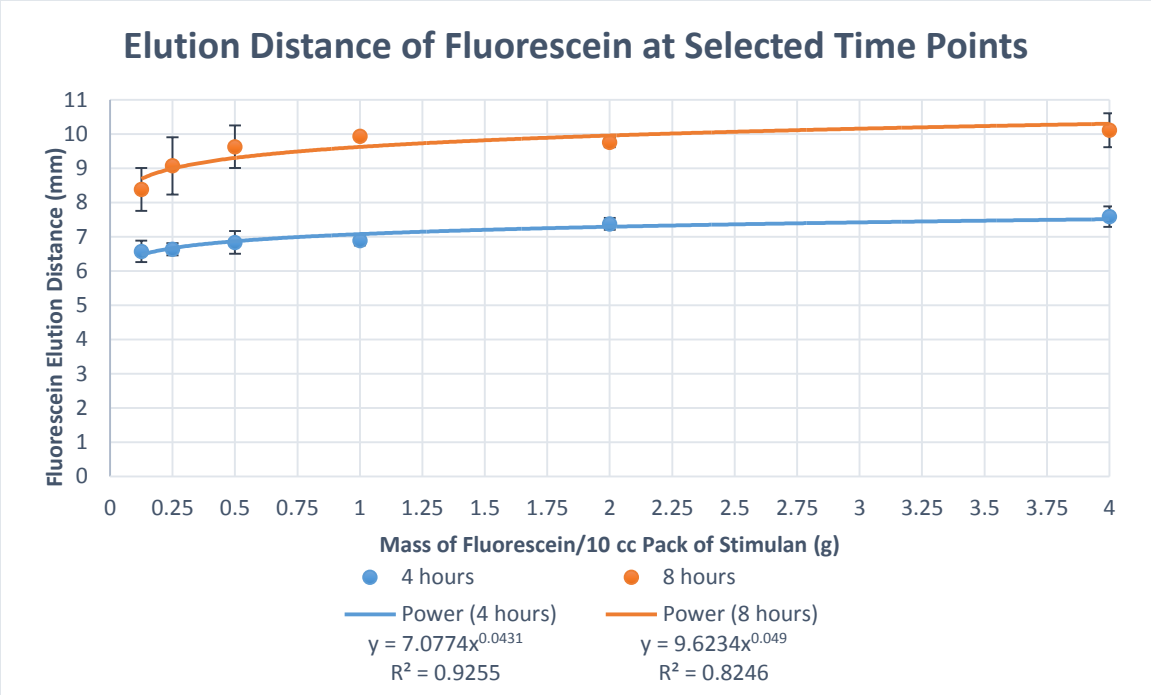


Figure 13: Plot of fluorescein elution distance from Stimulan beads as a function of concentration. The 4 and 8 hour time points through 1% Agar were shown. Each data point represent the mean of three trials.

In determining if a relationship between the elution distance of fluorescein from Stimulan beads and the bead's ultimate compressive strength exists, Figure 14 was created. From the slopes of the linear best fit trend lines for the middle and final hours of the time lapse, no significance was determined between altering the microstructure of a Stimulan bead and a decreased elution efficacy.

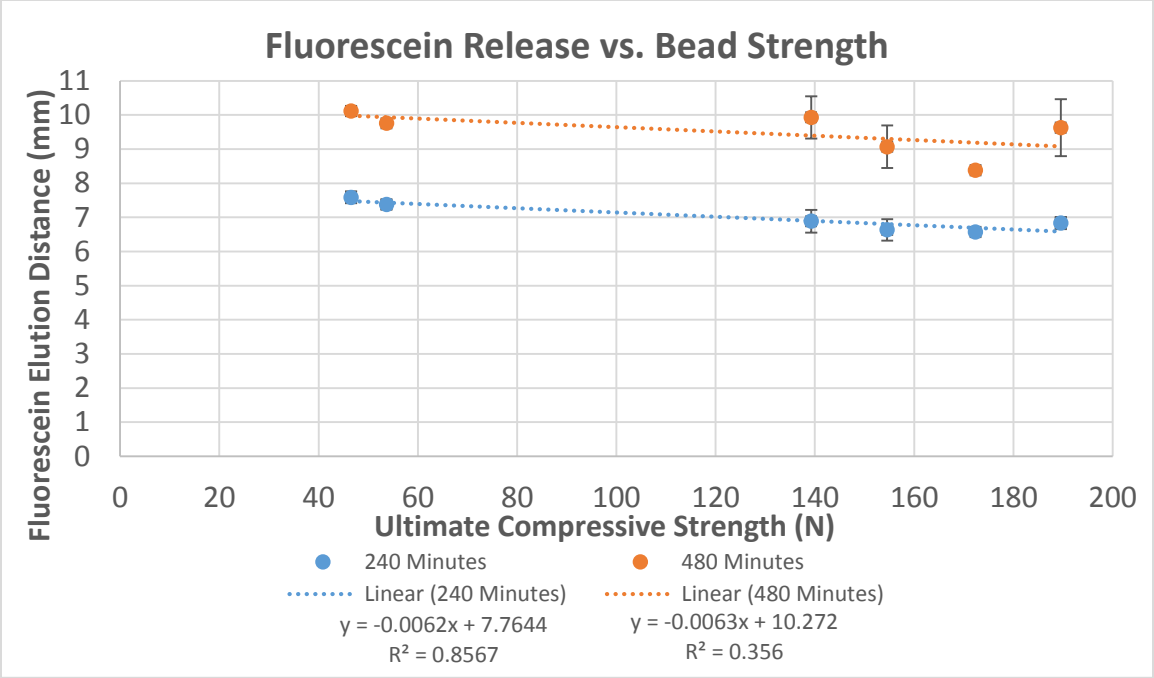


Figure 14: Linear relationship of fluorescein elution distance as a function of ultimate compressive strength. It was determined that no relationship exists. Each y and x coordinate, respectively was the mean of the three and six trials.

3.4 Elution through Bacterial Lawn

After plates of *P. aeruginosa* lawns were grown for 24 hours, fluorescein-impregnated Stimulan beads were placed in their center, as well as in the LB Agar growth media. Their elution distances were imaged by a gel documentation UV transilluminator camera. 15 minutes passed before all beads were placed on their respective plates and imaged. Hourly time points of the elution of a Stimulan bead through a *P. aeruginosa* lawn were comprised into Figure 15. It was clear that with an increase in time, the elution

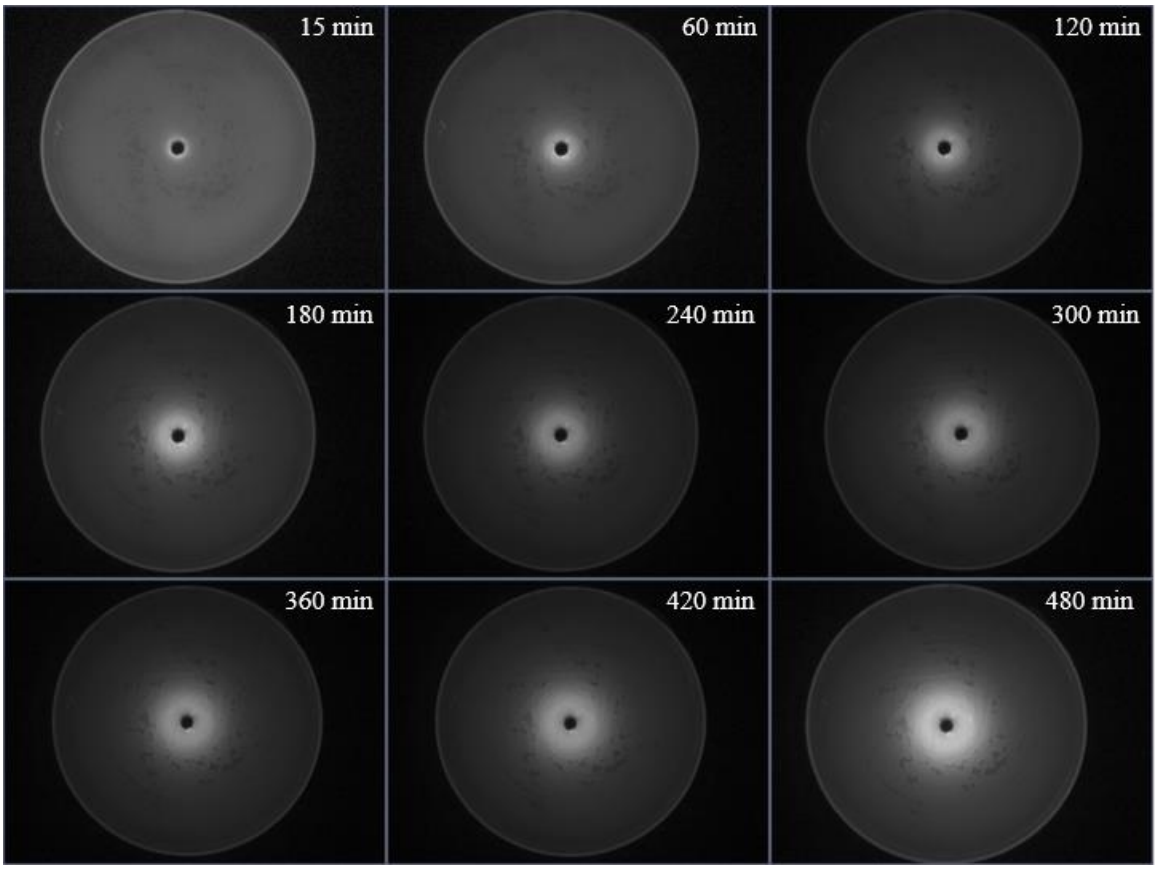


Figure 15: Images taken from the Gel Documentation system of the elution of fluorescein from a Stimulan bead through a *P. aeruginosa lawn*. The images depict the time lapse of a Stimulan bead with 1 g of fluorescein per 10cc pack. The UV camera was capturing the elution area from the reflection of UV rays off of the fluorescein. Also, the UV camera captured reflections from the auto-fluorescence of *P. aeruginosa*, thus distorting the imaging. As a result, plotting the elution was difficult, and had to be performed image by image in Fiji.

distance of the fluorescein across the lawn increased.

The elution distances across the lawn and control media were quantified in ImageJ, and plotted as a function of time for each concentration (Figure 16). Because *P. aeruginosa* is auto-fluorescent, Fiji was unable to stack the images as in the previous experiments. As a result, the images were each given a different automatically calculated sensitivity value for fluorescein intensity. Images with the least variable lawn reflection

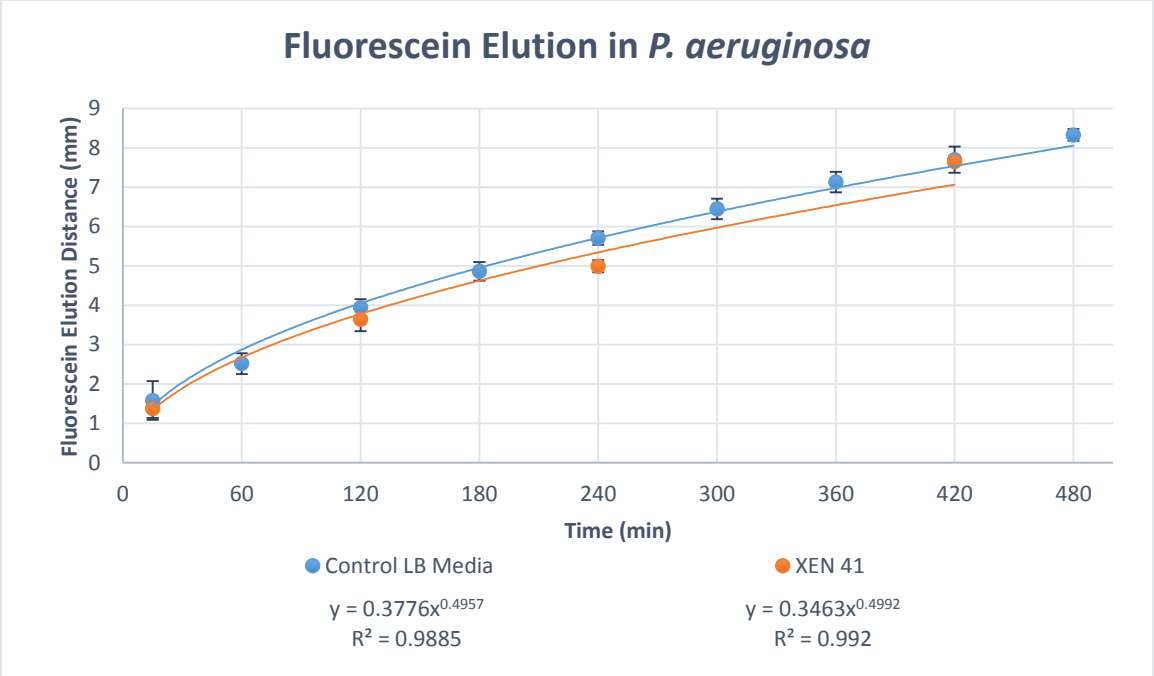


Figure 16: Plot of elution distance of fluorescein as a function of time through a pre-grown *P. aeruginosa* lawn and a control lawn. Each data point represents the mean of three trials. Power law trend lines were used represent the data because it should adhere to Fick's second law of half order kinetics.

were qualitatively selected, analyzed, and plotted—15, 120, 240, and 420 minutes. Power law trend lines were implemented in representing the data because the elution profiles were compared to the half order release rate kinetics in Eq. 2. A statistically significant difference in elution distance through a bacterial lawn was not observed (Table 2); thus,

CONTROL VS. XEN 41 ELUTION

Time (min)	P
15	0.276
120	0.128
240	0.0173
420	0.413

Table 2: P-values calculated by comparing different concentrations of fluorescein-impregnation of Stimulan beads and the non-impregnated fluorescein beads. Although at 240 minutes, the elution distance of fluorescein was significantly less through the lawn, the first and last time points showed no significant difference, thus it cannot be concluded that the lawn inhibited fluorescein's elution.

the transport through the lawn was not significantly different than transport through the agar, supporting the use of the agar release method to understand how the kinetics of antibiotic release may kill pathogenic biofilms.

4. Discussion

4.1 Effect of Additives on Bone Cement Microstructure and Orthopaedic Applications

The capability of bone cement to maintain its structural integrity and elution ability upon impregnation was analyzed because it is not well understood. Surgeons implement their own recipes for mixing bone cements in the operating room, and depending on what concentration of additive is added, the microstructure of the beads have been shown to be significantly altered. When PMMA was impregnated with one antibiotic (Figure 8), it showed significant weakening in its first crack strength. Furthermore, in applications in which the surgeon is unable to identify the type of bacterial infection present in a patient, impregnating both tobramycin and vancomycin, resulted in an even weaker microstructure of PMMA. This is especially concerning because PMMA cements serve a structural role when antibiotic-impregnated and placed at the site of total joint arthroplasty. Spacers are created with antibiotic-loaded PMMA cement to maintain a normal alignment and spacing of a joint.[xv] The antibiotic-loaded cement is also implemented to adhere an implant to the surrounding bone to maintain stability of the joint. In both of these applications, the antibiotic-loaded cement could be subject to the first crack load shown in Figure 8. For example, when both antibiotics were used, the first crack was observed at 377.57 (±87.25) N, which is only about 85 lbs. Although the size and shape of a spacer and a cement used for adhesive purposed would

be larger and different than that of a bead, sections of the structures could certainly exhibit plastic deformation and cracking over their life spans.

In Figure 7 it was determined that impregnating Stimulan beads with one or two antibiotics did not significantly alter its ultimate compressive strength. Contrastingly, in Figure 9 there were significant alteration in the microstructure of Stimulan. This is attributable to mixing difficulties between the various batches. When Stimulan was antibiotic-loaded, a whole pack of Stimulan was used to mix these beads and was spread across the entire mat. However, in impregnating Stimulan with varying concentrations of fluorescein, the packs were divided into tenths of packs to conserve materials. As a result, the bead density after spreading across the mat was less uniform and dense than when a whole pack was used.

When the lower concentrations of fluorescein—(0.125 g, 0.25 g, and 0.5g per 10cc pack of Stimulan—)were introduced into the Stimulan microstructure, there was an initial increase in ultimate compressive strength. Stimulan is a purified, ionically bonded powder that is hardened by recrystallization into gypsum. For concentrations of 2 g and 4 g, because the Stimulan powder, and not the fluorescein, is transformed into a paste to be spread from the addition of the liquid, the difficulty of mixing and the amount of impurities in the microstructure is exceptionally high. This is seen Figure 9, in which the Stimulan becomes increasingly brittle and harder to mix, thus losing density and purity, and forming weaker beads. Dissimilarly, because PMMA is comprised of a large polymer chain, any additive would have inherently weakened the microstructure due to pores and breaks in the chain.

In both figures, standard deviations were exceptionally large, further proving the difficulty of mixing Stimulan upon impregnation. Interestingly, when infusing 1 g of fluorescein into Stimulan beads—the suggested amount of powder impregnation by Biocomposites, Ltd.—there was not a statistical difference in ultimate compressive strength (Figure 9).

4.2 Elution Kinetics of Stimulan

The elution ability of Stimulan is already well understood;[xvi] however, previous studies were only conducted using the 1 g of powder antibiotic per 10cc pack recommendation. Because surgeons frequently vary the concentration of antibiotic in Stimulan, it is more clinically relevant to study elution ability as a function of time and concentration. It is clear in Figure 11 that upon increasing the concentration of fluorescein in a Stimulan bead, the elution rate is increased. However, this increase is depicted in Figure 12 to be miniscule, and may present more cytotoxicity issues than it is worth. At additive concentrations of 2 g and 4 g per 10cc pack of Stimulan, the elution rate begins to level off in what appears to be a power law relationship.

The equations for the trend lines of additive concentrations of 0.25 g and 0.5 g, exhibited identical exponents (0.425). From manipulating Eq. 1, it can be determined that the coefficients of the power law curves in Figure 11 are equivalent to the square root of two times the diffusion coefficient. The fluorescein not only diffuses through the bead, but also the 1% Agar. The diffusion coefficient of the agar is constant at all concentrations of fluorescein. Thus, when the coefficients of the power curve equations change, from Eq. 1, the diffusion coefficient of the bead must change. In analyzing the

increase in coefficients from 0.25 g (0.663) to 0.5 g of impregnation (0.688), it is clear that the diffusion coefficient increased, and thus the porosity did as well. Interestingly, this increase in porosity actually increased the strength of the bead. Furthermore, because increased impregnation did not alter the elution ability of the Stimulan beads relative to the higher concentration, and statistically irrelevant slopes were present in Figure 14, it is clear that the elution distance of an additive from a Stimulan bead has no relationship to its mechanical strength, contrary to the hypothesis. However, because the diffusion coefficient of the Stimulan beads increased, the hypothesis of an increased concentration effecting elution efficacy held true.

Although the short term release kinetics of fluorescein-impregnated Stimulan can be relatively aligned with antibiotic release, simply measuring the elution distance of fluorescein in 1% Agar growth media, would not be physiologically relevant due to the presence of biofilm. Although fluorescein diffusion through a biofilm was previously proven to exhibit a lower diffusion coefficient than 1% Agar, the hypothesized decreased in elution distance through a lawn was not significant when implementing an LB Agar growth media control (Figure 16).

According to the Einstein-Smoluchowski relationship, unsteady state diffusion is given by distance as a function of the square root of time. Because, mathematically, fluorescein diffusion through a growth media holds true to this relationship, power law trend lines were plotted on the elution distance graphs. As was made clear in Figures 11, 13, and 16, the half order kinetics were not exactly followed. In this study, the fluorescein had to not only diffuse through a growth media, but it also diffused through the Stimulan

bead itself. If the fluorescein was administered to the growth media without a reservoir, the trend lines would have been expected to be more closely aligned with a half order elution distance.

5. Conclusions and Future Works

Antibiotic-infused bone cements are promising materials in fighting and preventing infection after total joint arthroplasty procedures. Furthermore, impregnating the cements with an appropriate quantity of antibiotics can enhance their efficacy. Over medicating can be an easy solution to fighting infection; however, surgeons must understand that the original application of the beads could be seriously hindered. Although the elution ability of Stimulan was not significantly hindered upon increased impregnation, its ultimate compressive strength was. Furthermore, PMMA, implemented for its strength, was shown to crack at a significantly lower load upon impregnation. A possible alternative to over concentrating bone cements with antibiotic would be to increase the quantity of cement, while keeping the overall quantity of antibiotic constant. Finally, although the study proved the use of Agar release was a viable method for understanding the kinetics of antibiotic through a biofilm, the analysis of the results were difficult due to the autofluorescent behavior of *P. aeruginosa*. By implementing a different bacterial strain, more conclusive results may be obtained.

Our group intends to further study the efficacy of PMMA and Stimulan beads in orthopaedic applications. PMMA will be studied to determine, similarly to Stimulan, if its first crack strength would be hindered by higher additive concentrations. Furthermore, for more conclusive evidence on if an alteration in microstructure occurred in the beads, SEM images will be captured after their impregnation. According to the results in this

study, the microstructure was deformed in some manner due to the beads' decrease in strength. Finally, fluorescein-labeled vancomycin will be impregnated into Stimulan to determine if it elutes through Agar at a statistically similar rate to fluorescein-only. Confirming if vancomyin elutes similarly to fluorescein will allow researchers to utilize fluorescein—a cost-effective alternative to antibiotic—in their elution studies.

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[vii] Refer to [ii]

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[xv] http://orthoinfo.aaos.org/topic.cfm?topic=A00629

[xvi] Cooper et al. Antibiotic stability in a synthetic calcium sulphate carrier for local delivery. Poster presented at European Bone and Joint infection Society Annual Meeting, Prague, Czech Republic, 2013 - See more at: http://www.biocomposites.com/our-products/stimulan/#sthash.ZdgSJ1KY.dpuf

Appendix

Figure A1 (calculation): One pack of 10 cc Stimulan includes 26 g of materials, which after setting and evaporation, forms 23.76 g of cement. One pack of Palacos® R includes 59.6 g of materials, which after setting and evaporation, forms 53.17 g of cement. When 1 g of powdered antibiotic is mixed into a Stimulan pack, each bead will be infused with 4.2 wt% of antibiotic. For PMMA beads, 4.2 wt% of antibiotic per bead is a weight of 2.23 g per pack of Palacos® R.

```
%% Modeling Fluorescein Elution with Respect to Time and Bead Concentration
% Created by NRF: 04/05/2015
% Last Modified by NRF: 04/07/2015
% Clear All
clc; clear all

%% List Data Points in Coordinate Form
% x-coordinates (time in hours)
x=[0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8
    0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8 0 1 2 3 4 5 6 7 8];
% y-coordinates (concentration of fluroescein in grams per pack of
% Stimulan)
y=[0 0 0 0 0 0 0 0 0 0.125 0.125 0.125 0.125 0.125 0.125 0.125 0.125 0.125
    0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.25 0.5 0.5 0.5 0.5 0.5 0.5
    0.5 0.5 0.5 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 4 4 4 4 4 4 4 4 4];
% z-coordinates (Elution Distance in mm)
z=[0 0 0 0 0 0 0 0 0 0 3.883 5.308 5.660 6.571 7.545 8.365 8.711 8.383 0
    3.715 4.778 5.485 6.636 7.631 8.593 9.237 9.070 0 4.199 4.833 6.333
    6.835 7.763 8.516 9.077 9.627 0 4.166 4.692 6.208 6.888 7.877 8.501
    9.255 9.931 0 3.833 5.329 6.478 7.376 8.305 9.061 9.447 9.757 0 3.976
    5.564 6.704 7.588 8.389 9.189 9.577 10.114];

%% Graph Elution Profile with Mesh
% Set-up Profile in terms of x and y (time and concentration)
tri = delaunay(x, y);
% Plot Mesh
trisurf(tri, x(:), y(:), z(:));
% Label Axis
xlabel('Time of Elution (h)');
ylabel('Concentration/10cc Stimulan (g)');
zlabel('Elution Distance (mm)');
```

Figure A2: MATLAB script that codes for 3D mesh of elution distance from a Stimulan bead as a function of time and fluorescein concentration. Time, concentration, and elution distance are represented by x, y, and z respectively.

FORMULA FOR VARYING AMOUNT OF FLUORESCEIN IN STIMULAN BEADS	
One pack Stimulan Powder (g)	20.00
One pack Stimulan Liquid (mL)	6
One pack Stimulan (g)	26.00
One pack set Stimulan (g)	23.76
Fluorescein per pack (g)	1.00
Total weight of bead set (g)	24.76
Average weight per bead Fluor in Stim (g)	0.097
Number of beads desired for batch	50.00
Weight of number of beads desired (g)	4.8375
Percentage of Materials needed to make desired batch size after setting	19.5%
Weight of Stimulan Powder Needed (g)	3.908
Weight of Stimulan Liquid Needed (µL)	1172
Weight of Fluorescein Needed (mg)	195.4

FLUORESCEIN WEIGHT TABLE			
Fluor/Pack (g)	**Weight of Mix (g)**	**Beads/1 Pack**	**Fluor/Bead (mg)**
0.000	23.76	245	0.00
0.125	23.89	246	0.51
0.250	24.01	248	1.01
0.500	24.26	250	2.00
1.000	24.76	255	3.92
2.000	25.76	266	7.53
4.000	27.76	286	13.98

Table 1A and 2A (top and bottom, respectively): Calculation of the necessary materials on one pack of Stimulan required to formulate a desired number of beads with a desired concentration. The weight of the number of beads (0.097 ± 0.004 g/bead) desired divided by the overall weight of one set pack of Stimulan cement was calculated as a percentage. This percentage was multiplied by each of the three necessary materials to formulate fluorescein-impregnated Stimulan beads. The example shown is for 1 g per 10cc pack of Stimulan. The highlighted concentration is recommended by Biocomposites, Ltd.